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14. ABSTRACT

We propose that AR phosphorylation at serines 213 and 650 regulate differential target gene expression and recruitment to gene promoters via altered interaction with other cellular transcription factors. To test this hypothesis we have conducted yeast two-hybrid analysis with the N-terminus of wild type AR as well as AR S213A and AR S213E variants. Our preliminary analysis indicates that the screen is preferentially isolating proteins with a known role in gene transcription and we are currently assessing the phosphorylation-dependence of the putative AR interacting proteins. Additionally, we have generated PC3 cells stably transfected with wild type, S650A and S650E AR. We have shown that the cells activate endogenous target genes in response to androgens and are currently investigating classes of genes affected by differential AR phosphorylation. Further, we have optimized conditions to isolate pools of hyper- and hypo-phosphorylated AR in order to isolate proteins that interact with AR in a phosphorylation-dependent manner. These proteins will be identified via mass spectrometry.

15. SUBJECT TERMS
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Function of an Androgen Receptor Coactivator Regulated in Prostate Development and Prostate Cancer

INTRODUCTION:

The androgen receptor regulates prostate cell growth and differentiation and plays a critical role in prostate cancer progression. Like other steroid hormone receptors, AR is a phospho-protein and phosphorylation is believed to regulate AR function. To provide insight into the regulation and function of AR phosphorylation, we generated novel antiserum that specifically recognizes AR phosphorylated on key serine residues. Utilization of these antibodies indicates that AR phosphorylation is tightly regulated in urogenital developmental and in differentiated adult prostate. Thus, the development of AR phosphorylation site-specific antibodies along with AR mutant molecules provides a unique opportunity to study the regulation of AR phosphorylation by cellular kinases as well as the impact of phosphorylation on AR function.

Using our novel anti-serum that specifically recognizes AR phospho-serine 213 (P-S213), a putative site of Akt phosphorylation, we demonstrated rapid phosphorylation at S213 in response to agonists R1881 and DHT, but not in response to antagonists bicalutamide or flutamide. By immunohistochemistry, the AR-P-S213 antigen was detected in prostate epithelial but not stromal cells despite the fact that an antibody recognizing both phosphorylated and non-phosphorylated forms of AR demonstrates that AR is present in both cell types as expected. In fetal tissue, the AR-P-S213 antigen was present in epithelial cells of the urogenital sinus when endogenous androgen levels are high, but absent at a later stage of development when endogenous androgen levels are low. Immunoreactivity is evident in differentiated cells lining the lumen of the urogenital sinus, but not in rapidly dividing, Ki67 positive cells within the developing prostate or stromal tissue, suggesting that site-specific phosphorylation of AR S213 by cellular kinases occurs in a non-proliferating cellular milieu. The exquisite cell type specificity of AR S213 phosphorylation suggests that phosphorylation is tightly regulated by cellular kinases and may function in AR-mediated transcription in a specified cellular context.

Examination of phosphorylation of AR at serine 650 (S650) was also conducted. Interestingly, phosphorylation of S650 is enhanced by treatment with forskolin (FSK), Epidermal Growth Factor (EGF) and phorbol-12-myristate-13-acetate (PMA)[Gioeli, D., J. Biol. Chem., 2002] suggesting that AR phosphorylation may be intricately linked to signal transduction processes regulating tumor promotion and cell growth. Consistent with the idea that multiple cell signaling pathways contribute to phosphorylation at AR S650, we find enhanced phosphorylation of AR S650 following R1881 treatment using antibody against AR phospho-serine 650. Characterization of signaling pathways that contribute to phosphorylation of AR S650 is in progress.

BODY

The original grant outlined two tasks in the statement of work. Each task is listed below followed by a description of the research progress relevant to the task.

Task 1. Determine the effect of phosphorylation on AR-mediated gene transcription. Transcription of AR target genes will be compared in HPr-1 prostate cell lines stably expressing wild type AR versus phosphorylation site mutants.

Although the goal of aim 1 is to learn more about AR signaling as it relates to prostate biology, our ultimate objective is to understand and circumvent prostate cancer signaling pathways. Therefore, although the aim originally proposed to utilize an immortalized, non-transformed cell line, HPr-1, we decided that it would be better to use metastatic PC3 cells. Our initial reservation about using PC3 cells was that AR target genes might not be normally activated in these cells upon co-transfection with AR variants and androgen stimulation. Since HPr-1 cells are fairly difficult to transfect, we decided to create and clone the AR phosphorylation-site specific variants into a retrovirus vector that also contains a fluorescent GFP marker. Our reasoning was that we could first test the constructs in PC3 cells to see if endogenous androgen-responsive genes were activated and if not we would still have the option to use HPr-1 cells. Therefore wild type AR, AR S650A and AR S650E were inserted in the retrovirus vector and used to stably infect PC3 cells. GFP positive pools of cells were selected in the cell sorting facility. To determine if endogenous genes were activated in response to androgens, cells were steroid starved prior to treatment with the synthetic androgen, R1881, and real time PCR was conducted on selected AR targets, PSA, FKBP5 and F5.

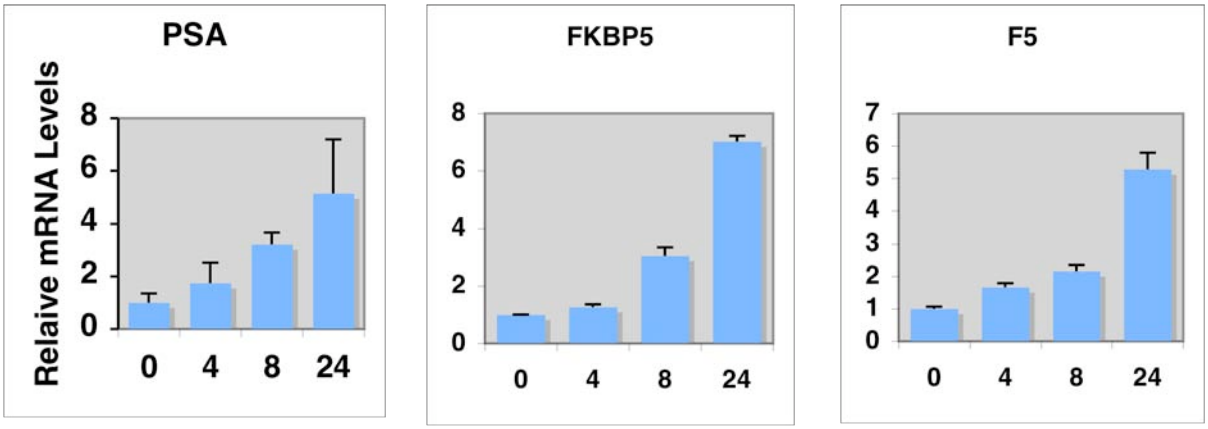


Figure 1. Analysis of AR target gene expression in stably transfected PC3 cells. The figure shows relative mRNA expression of PSA, FKBP5 and F5 as measured by real time PCR.

The results indicate that AR target genes PSA, FKBP5 and F5 are activated in a time dependent manner following androgen treatment, strongly suggesting that PC3 cells are suitable for the studies proposed in this aim. We are currently conducting real time PCR on the androgen-treated pools of PC3 cells infected with either wild type AR, AR S650A and AR S650E to see if there is variation in target gene expression in wild type AR compared to the phosphorylation site mutants. The preliminary results are promising and it appears that there are differences in individual target genes depending on S650 phosphorylation. Whether or not this is the case we will then proceed to an unbiased approach doing genome wide analysis via gene expression array to see if phosphorylation plays a role in determining the functional classes of genes regulated via androgens.

Task 2. Examine the effect of AR phosphorylation on recruitment of coactivators and corepressors to the androgen-regulated PSA promoter.

The previous report demonstrated that we could detect robust androgen-dependent AR recruitment to the PSA promoter via chromatin immunoprecipitation assays. Since then, we have conducted similar experiments with the phospho-antibodies. The results indicated that AR S-213 and AR-S-650 are not strongly recruited to the PSA promoter in an androgen-dependent manner in LAPC4 cells. We conducted these experiments in LAPC4 cells since our published findings showed that endogenous AR S213 is phosphorylated in LAPC4 cells. However, we do not yet know if the PSA gene transcription is strongly affected by AR phosphorylation. Therefore, this experiment will be repeated with promoters of additional AR-driven genes when the target genes from specific aim 1 have been identified.

Task 3. Characterize proteins that interact with AR S213 and S650 in a phosphorylation dependent manner via yeast two-hybrid analysis.

We have undertaken several approaches to examine proteins that interact with the androgen receptor in a phosphorylation-dependent manner. Briefly, we have conducted yeast two hybrid to analyze proteins that interact with AR-P-S213 and we are performing immunoprecipitation followed by mass spectrophotometry to analyze proteins that interact with AR-P-S650.Two different approaches were taken because the phosphorylation sites are in different regions of the AR and within different domains of the receptor. Serine 213 is within the 488 amino acid N-terminal region of the AR. This region has been used successfully for yeast two-hybrid screening previously so we felt that this approach will also be effective to identify proteins that bind to the N-terminus of AR in a phosphorylation-site specific manner. Approximately 45 clones have been isolated from yeast, transformed into bacteria and sequenced. Most of these contain identifiable proteins in BLAST searches. Clones that consisted of vector only or of short peptide sequences were discarded. All interesting clones have been re-transformed into yeast to verify the original result. Of the clones with which we have preceded the farthest, 8 out of 12 of the corresponding proteins have a documented role in transcription giving credence to the quality of the screen.

We are currently comparing the β-gal expression induced by the interaction of library proteins with AR_{1-488} S213E *vs.* AR_{1-488} S213A to identify proteins that associate with AR in a manner dependent on phosphorylation. We have identified several promising candidates and follow up experiments are in progress.

The strategy to strategy to identify proteins that interact with AR-P-S650 is outlined below. Serine 650 is located in the hinge region, a small unstructured region that lies between the DNA binding domain and the ligand-binding domain. Since this region is only about 40 amino acids we were concerned that it might not fold properly in the yeast two-hybrid assay. We decided to perform immunoprecipitation followed by mass spectrophotometry to identify protein that bound to this region. Although we have made phosphorylation site-specific antibodies that recognize AR-P-S650 we do not wish to use these antibodies for immunoprecipitation since the antibodies themselves may obscure interaction with associated proteins. To circumvent this problem we optimized conditions under which AR-P-S650 is robust and under which it is inhibited. As published previously, we were able to show that AR is robustly phosphorylated upon PMA treatment (Figure 2, below). In addition, we were able to identify inhibitors of the P38 and JNK kinase pathway that could interfere with S650 phosphorylation.

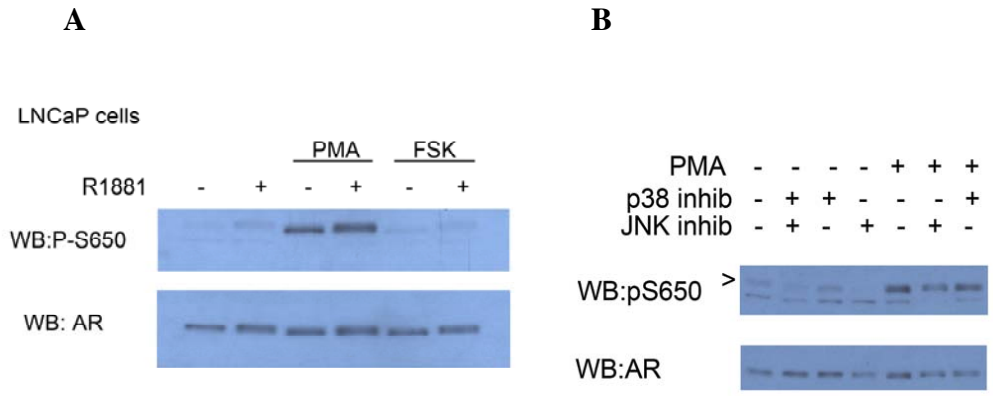


Figure 2: **A** PMA but not R1881 or Forskolin induces S650 phosporylation. LNCaP cells were steroid starved and treated for 2 hrs with 10nM R1881 (lanes 2, 4 and 6), 1uM PMA (lanes 3 and 4) or 50uM FSK (lanes 5 and 6). 50 ug of whole cell lysate were run per treatment. **B** JNK and p38 reduce both basal and PMA induced S650 phosphorylation in LNCaP cells. PMA stimulates the stress kinase pathway, which includes the kinases JNK and p38. Inhibition of these kinases significantly reduces basal S650 phosphorylation (lanes 2,3 and 4). These inhibitors are less potent against PMA treated cells (lanes 6 and 7). Cells were pretreated with inhibitors at concentrations above for 30 minutes. Cells were then left with inhibitors or treated with 20nM PMA and inhibitors for an additional 2 hours.

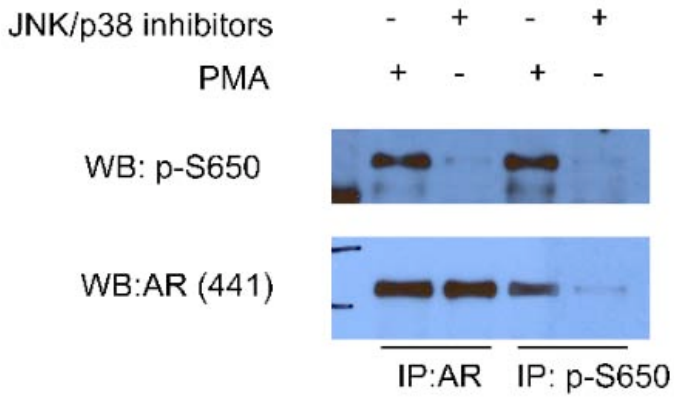
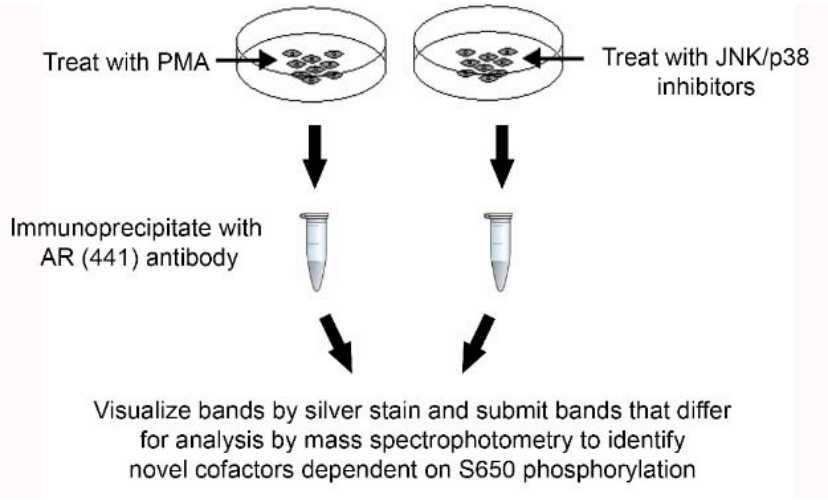


Figure 3: Separation of non-phosphorylated AR and S650 phosphorylated AR using PMA or inhibitors followed by immunoprecipitation. LNCaP cells were steroid starved and then treated with either 20nM PMA or 25 uM JNK inhibitor and 50uM p38 inhibitor for 2 hr. 1 mg of total lysate was immunoprecipitated with either AR (441) antibody (lanes 1 and 2) or pS650 antibody (lanes 3 and 4).

On the basis of these experiments we are confident that we will be able to isolate immunoprecipitated pools of AR that are hypo- or hyper-phosphorylated on S213. Therefore we have begun to treat LNCaP cells as outlined in the diagram below and are currently optimizing the conditions based on differences in the silver stained gels upon the different treatments.



KEY RESEARCH ACCOMPLISHMENTS

- A reverse yeast two-hybrid screen has identified multiple proteins that interact with the AR phosphorylation-site mimetic S213E
- The identity of a dozen proteins has been confirmed so far and the majority play a documented role in gene transcription
- Stably infected PC3 cells have been made and we have shown that target gene expression in response to androgen is normal in the cells.
- Initial experiments conducted with the PC3 cell lines indicates that AR target gene expression is altered in response to phosphorylation.
- Conditions were optimized to isolate pools of hyper and hypo-phosphorylated androgen receptor for analysis of associated proteins.

REPORTABLE OUTCOMES

Abstracts and Publications, 2006-2007

Susan Ha, Rachel Ruoff, Hong Ying Huang, Ellen Shapiro, Michael J. Garabedian, Samir S. Taneja and Susan K. Logan Cell specific regulation of androgen receptor phosphorylation *in vivo*. Keystone Meeting, Steroid Sisters, 3/18/2006, abstract

Rachel Ruoff, Susan Ha, Hong Ying Huang, Ellen Shapiro, Michael J. Garabedian, Samir S. Taneja and Susan K. Logan. Regulation of androgen receptor phosphorylation in prostate cancer. Cold Spring Harbor Meeting. 11/1/2006, abstract

Rachel Ruoff, Susan Ha, Hong Ying Huang, Ellen Shapiro, Michael J. Garabedian, Samir S. Taneja and Susan K. Logan. Regulation of androgen receptor phosphorylation in prostate cancer. Society of Biological Urology Research, Tucson, Arizona, 11/16/06, abstract

Nwachukwu, J.C., Li, W., Huang, H.Y., Ruoff, R., Shapiro, E., Taneja, S., Logan, S.K. and Garabedian, M.J. Transcriptional regulation of the androgen receptor cofactor, ART-27. Mol Endocrinol. 2007 Dec;21(12):2864-76.

Shapiro, E., Huang, HY., Ruoff, R., Lee, P., Tanese, N. and Logan, S.K. Regulation of the HP1 family in prostate development and cancer. In press. J. Urology 2008

Research training during the period of grant support (under my immediate supervision):

Raluca Pancratof	Sackler rotation student	1/15-06-4/14/06
Andrew Hanover	NYU undergraduate	1/30/06-5/29/06
Derick Mitchell	postdoctoral fellow	9/1/06-7/16/06
Shivani Garapaty	graduate student readings, weekly	3/19/07-5/31/07
Kyle Monk	SURP summer program	6/1/07-8/31/07
Neha Kaul	Sackler rotation student	4/11/07-7/10/07
Paolo Mita	Sackler student	4/11/07- presesnt
Lindsey DeCarlo	Sackler rotation student	9/18/07- 12/20/07
Julia Staverofsky	Sackler rotation student	5/11/07-present

In advisory function (thesis committee):

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Jerome Nwachukwu	Ph.D. candidate-	Michael Garabedian, advisor
Pricilla Maldonado	Sackler student-	Jim Borowiec, advisor
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Shivani Garapaty	Sackler student	Herb Samuels, advisor
Paolo Mita	Sackler student	Susan Logan, advisor
Julia Staverofsky	Sackler student	Susan Logan, advisor
Kay Yeung	MD/PhD candidate	Herb Samuels, advisor

CONCLUSION:

We propose that AR phosphorylation at serines 213 and 650 regulate differential target gene expression and recruitment to gene promoters via altered interaction with other cellular transcription factors. To test this hypothesis we have conducted yeast two-hybrid analysis with the N-terminus of wild type AR as well as AR S213A and AR S213E variants. Our preliminary analysis indicates that the screen is preferentially isolating proteins with a known role in gene transcription and we are currently assessing the phosphorylation-dependence of the putative AR interacting proteins. Additionally, we have generated PC3 cells stably transfected with wild type, S650A and S650E AR. We have shown that the cells activate endogenous target genes in response to androgens and are currently investigating classes of genes affected by differential

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